REMARKS/ARGUMENTS

I. Status of the Claims

Claims 1-26, 28, and 29 are pending. Claims 6-10 are withdrawn from consideration as drawn to a non-elected invention. Claim 27 is canceled herein without prejudice and claims 28 and 29 have been added.

II. The Present Amendments

No new matter is added by the present amendments.

Claim 1 has been amended to recite that the subject to whom the inhibitor of soluble epoxide hydrolase (sEH) is administered does not have hypertension or whom is being treated for hypertension by an agent that is not an inhibitor of sEH. The recitation is supported throughout the specification, including page 10, lines 26-29. New claims 28 and 29 recite embodiments already within the scope of claim 1 as originally presented.

Withdrawn claim 6 is amended to conform its recitation of the enzyme inhibited to that of the other claims.

III. Response to the Office Action

Applicants acknowledge with appreciation the Action's withdrawal of a number of rejections contained in the previous Office Action. The present Action, however, imposes new rejections on the claims. Applicants amend in part and traverse all the rejections. For ease of reference, the rejections are addressed individually below in the order in which they are presented in the Action.

A. Rejection of the Claims as Anticipated by Erickson

Claims 1, 2, 18-20, and 27 are rejected under 35 U.S.C. § 102(b) as anticipated by Erickson, WO00/23060 ("Erickson"). According to the Action, Erickson teaches the administration of sEH inhibitors for the treatment of immunological disorders, including atherosclerosis. Action, at page 3. The Action acknowledges that Erickson is silent about the

methods claimed in the claims under examination, but argues that the methods of the claims under examination would inherently be present in administering sEH inhibitors in the methods taught in Erickson. *Id.* Applicants traverse.

Erickson asserts that sEH inhibitors can be used to treat some 70 autoimmune diseases and disorders associated with T-lymphocyte mediated immune function, as well as transplantation, allograft or xenograft rejection, and graft versus host disease. Among these diseases and disorders are the ones relied on by the Action as anticipating the claims under examination.

Erickson did not, of course, actually test sEH inhibitors in persons with any of these 70+ disorders and diseases, or in any animal models of these diseases and disorders. The basis for Erickson's broad assertion that sEH inhibitors can be used to treat autoimmune diseases and disorders associated with T-lymphocyte mediated immune function is a limited set of assays on 9 compounds which Erickson maintains show that sEH inhibitors also block the influx of calcium ions into Jurkat cells and block the production of IL-2 by human T-lymphocytes. From these findings, Erickson extrapolates that sEH inhibitors can be used to suppress T-lymphocyte mediated immune responses and, hence, the many conditions set forth in its laundry list. See, e.g., Erickson at page 6, bottom paragraph.

No doubt Erickson and his assignee hoped that the association that they believed present would prove out and that patients with one of the 70+ listed conditions would indeed benefit from the administration of sEH inhibitors. Unfortunately, this has not proved out. It is now known that some good sEH inhibitors do not inhibit IL-2 production and some bad sEH inhibitors do. Whatever is responsible for reducing IL-2 production cannot be correlated to sEH inhibition.

Some evidence of this can be seen from Erickson's own data. Table 3 of Erickson, on page 9, shows that for compound 2, it took 36 nM to inhibit sEH activity by 50%, and 422 nM to inhibit IL-2 production by 50% (the amount of a compound to inhibit an activity by 50% is called its "IC_{50}"). By contrast, for compound 3, it took 464 nM to inhibit sEH activity by 50%, or almost 13 times as much as was required for compound 2. But, it took only 190 nM of compound 3 to inhibit IL-2 production by 50%, or less than half as much as needed of

compound 2 to achieve the same effect. The same story is true for the assay of calcium influx into Jurkat cells. It should also be noted that it took only 55 nM of 4-phenyl chalcone oxide to inhibit sEH by 50%, but it took 3000 nM to inhibit Jurkat calcium influx by 50%, whereas compound 4, which needed more than 10 times as much as 4-phenyl chalcone oxide to inhibit sEH by 50%, was 7 times more effective at inhibiting Jurkat cell calcium influx. But, while each of the sEH inhibitors in Erickson's study did show some reduction in IL-2 production, studies since then have shown that other sEH inhibitors do not reduce IL-2 production.

In other words, the association between sEH inhibition and inhibiting cytokine production, which is the basis of Erickson's assertion for the treatment of the different conditions listed in the application, has not proved out. Perhaps this is the reason why a review of the legal status (INPADOC) of the application on the European Patent Office website (attached) reveals that the application was not entered into the national stage in Europe and apparently has not issued as a patent anywhere in the world.

On the face of Erickson, the Action might be correct that Erickson would show an anticipation of the claims stated. But, to be a proper anticipation, the reference must also be correct. Persons of skill reading Erickson as of the priority date would have been aware that the extrapolation on which the statements in Erickson were based had not proved out. Accordingly, Erickson is not a proper anticipatory reference for purposes of §102(b).

Reconsideration and withdrawal of the rejection are respectfully requested.

B. Rejection of Claim 27 as Anticipated by Hammock

The Action rejects claim 27 as anticipated under § 102(b) by Hammock, WO 00/48593. Claim 27 has been canceled without prejudice.

C. Rejection of Claims 1, 2 and 27 as Anticipated by Ingraham

Claims 1, 2 and 27 are rejected as anticipated under 35 U.S.C. § 102(e) by Ingraham, US2003/0022929 ("Ingraham"; Ingraham has since issued as a patent, but the published application will be referred to herein for consistency). Action, at page 4. According to the Action, Ingraham teaches the administration of sEH inhibitors represented by Formula I to a subject for the treatment of cardiovascular diseases including atherosclerosis, coronary artery disease, angina, ischemia, ischemic stroke, and renal disease. *Id.* The Action acknowledges that Ingraham is silent about the methods claimed in the claims under examination, but argues that the methods of the claims under examination would inherently be present in administering sEH inhibitors in the methods taught in Ingraham. *Id.* Applicants traverse.

Ingraham's teachings on the use of sEH inhibitors are this: that in vascular smooth muscle cells, EETs provoke signaling pathways that result in vasodilation, that impaired endothelium dependent vasorelaxation is a characteristic feature of the syndrome known as endothelial dysfunction, and that endothelial dysfunction plays a significant role in a number of pathological conditions, including the 11 listed in column 2. See, Ingraham at column 2, top paragraph. Thus, Ingraham presents a simple syllogism that increasing EETs will have a therapeutic effect.

The problem with this alleged teaching is that, despite the simple syllogism stated in Ingraham, the etiology and causation of these 11 diseases and disorders is not simple. Moreover, even endothelial dysfunction itself is a complex problem. Persons of skill in the art were aware as of the priority date that endothelial dysfunction that regulation of vasodilation and constriction by the endothelium relies on a balance of vasoconstictors and vasodilators, growth promoters and growth inhibitors, with nitric oxide and the rennin-angiotensin system being of particular importance. See generally, Pepine, Clin Cardiol, 20 (Supp II) (1997) (copy attached). For example, Luscher and Barton, Clin Cardio 20 (Supp II) II-3-II-10 (1997) (copy attached) shows at least 17 vasoactive mediators released by the endothelium, including angiotensin, acetylcholine, angiotensin-converting enzyme, adenosine diphosphate, adenosine triphosphate, bradykinin, cAMP/cGMP, endothelin-converting enzyme, L-arginine, endothelin-1, serotonin, superoxide, TGF-β, prostaglandin H_2, prostacyclin, endothelium-derived hyperpolarizing factor,

and nitric oxide. See, page II-4, Figure 1. Moreover, even these known vasoactive agents can have different effects depending on the particular vasculature in which they act: acetylcholine acts as a vasodilator in epicardial coronary arteries with intact endothelium, but as a vasoconstrictor in patients with coronary artery disease. See, Panza, Clin Cardio 20 (Supp II) II-26-II-33 (1997) at page II-26, right hand column (copy attached).

Thus, a person of skill reading Ingraham would be aware that more than a simple syllogism would be needed to anticipate that raising EETs would be beneficial in any of the conditions listed. Applicants respectfully submit that Ingraham is not a proper anticipatory reference for purposes of §102(b). Applicants further submit that, whether or not Ingraham anticipates those portions of claim 1 that pertain to treatment of persons who have had a heart attack, decreased circulation to the heart, or undergone angioplasty, all of which might be said to be related to coronary artery disease, it would not anticipate new claims 28 and 29.

Reconsideration and withdrawal of the rejection are respectfully requested.

D. Rejection of Claims as Obvious over Ingraham and Selmon

Claims 11-17 and 22-26 are rejected under 35 U.S.C. § 103(a) as obvious over Ingraham in view of Selmon, U.S. Patent No. 6,120,516 (Selmon). According to the Action, Ingraham does not teach the administration of a sEH inhibitor to someone who has had a heart attack, a coronary bypass, angioplasty, a stent in an arterial lumen, or a natural or synthetic vessel engrafted in an arterial lumen, and the administration of CDU by itself or in combination with EETs. The Action states that Selmon discloses that atherosclerosis is a major cause of coronary artery occlusions and that such occlusions are routinely treated by bypass graft surgery, angioplasty, or stents. Action, at page 6. According to the Action,

> [o]ne having ordinary skill in the art would have expected that the administration of soluble epoxide hydrolase inhibitors that has been known to be useful in the treatment of atherosclerosis . . . would provide therapeutic utility in [a] patient who has (had) a heart attack, coronary bypass, angioplasty, a stent in an arterial lumen or a natural or synthetic vessel engrafted. One would have been motivated to combine these

references and make the modification because they are drawn to [the] same technical fields."

Action, at page 7. Applicants traverse.

The teachings of Ingraham have been discussed in the preceding section. Selmon appears to have been added simply because it states the well known fact that persons with coronary occlusions are sometimes treated with bypass surgery or with stents, and notes that bypasses sometimes become occluded. Apparently, the Action intends that the reader infer from this that persons with bypasses or stents can be treated with sEH inhibitors to prevent restenosis. Selmon does not, however, offer any teaching that stents restenose, nor any teaching that bypasses or stents become occluded because of a process that could be affected by the administration of an sEH inhibitor. And the actual teachings of Selmon are beside the point - what Selmon is concerned with is a device for the mechanical removal of hardened, calcified tissue. See, Selmon at column 2, lines 25-27 and description of the invention. Simply put, the Action shows no logical nexus between Selmon and the contention that it would motivate one of skill to use sEH inhibitors to treat any of the conditions listed in the rejected claims.

The Action further alleges, with respect to claims 17, 21, 25 and 26, that Ingraham teaches that EETs have a similar activity to sEH inhibitors and that enhancement of EET concentration would have a beneficial effect in patients in whom endothelial dysfunction has a causative role. The Action alleges that the two references in combination make clear that sEH inhibitors and EETs have a similar activity and that the combination of active ingredients with the same character is merely the additive effect of each component.

Applicant respectfully note that the citation of Selmon in this argument adds nothing. Selmon does not mention sEH, sEH inhibitors, or EETs, and thus has nothing to add to Ingraham in connection with the combination of sEH inhibitors and EETs. Selmon is apparently cited only because it recites the word "bypass," which then is used to support the application of the rejection to engrafted vessels. Selmon does not offer any teaching that stents restenose, nor any teaching that bypasses or stents become occluded because of a process that could be affected by the administration of an EET. Applicants respectfully maintain that the Action shows no

logical nexus between Selmon and the contention that it would motivate one of skill to use EETs inhibitors to treat any of the conditions listed in the rejected claims

Applicants also take this opportunity to clarify that EETs are not considered beneficial in the absence of administration of an exogenous sEH inhibitor, since they would be expected to be hydrolyzed by endogenous sEH too quickly to have beneficial effects. It is believed, as noted in the present specification, that the increased levels of endogenous EETs made possible by the administration of an sEH inhibitor, and the further increase possible with administration of exogenous EETs, that are reflected by the decreased proliferation of vascular smooth muscle cells in the studies reported in the specification.

Reconsideration and withdrawal of the rejection are respectfully requested.

E. Rejection of Claims as Obvious over Ingraham

Claims 3-5 are rejected under § 103(a) as obvious over Ingraham. According to the Action, Ingraham teaches the use of urea and carbamate inhibitors of sEH, and that their use in the claimed methods would be obvious over those teachings. Applicants traverse.

As pointed out in some detail above, persons of skill in the art were aware as of the filing date of the considerable complexity of the regulation of the balance between vasocontrictors and vasodilators in the epithelium. As noted above:

> regulation of vasodilation and constriction by the endothelium relies on a balance of vasoconstictors and vasodilators, growth promoters and growth inhibitors, with nitric oxide and the rennin-angiotensin system being of particular importance. See generally, Pepine, Clin Cardiol, 20 (Supp II) (1997) (copy attached). For example, Luscher and Barton, Clin Cardio 20 (Supp II) II-3-II-10 (1997) (copy attached) shows at least 17 vasoactive mediators released by the endothelium, including angiotensin, acetylcholine, angiotensin-converting enzyme, adenosine diphosphate, adenosine triphosphate, bradykinin, cAMP/cGMP, endothelin-converting enzyme, L-arginine, endothelin-1, serotonin, superoxide, TGF-β, prostaglandin H_2, prostacyclin, endothelium-derived hyperpolarizing factor, and nitric oxide. See, page II-4, Figure 1. Moreover, even these known vasoactive agents can have different effects depending on the

particular vasculature in which they act: acetylcholine acts as a vasodilator in epicardial coronary arteries with intact endothelium, but as a vasoconstrictor in patients with coronary artery disease. See, Panza, Clin Cardio 20 (Supp II) II-26-II-33 (1997) at page II-26, right hand column (copy attached).

Applicants maintain that persons of skill reading Ingraham would be aware of these complex relationships and would not have been motivated by Ingraham to use sEH inhibitors to treat any of the complex conditions recited in Ingraham. Accordingly, Applicants maintain that the Action fails to set forth a proper prima facie case of obviousness.

Reconsideration and withdrawal of the rejection are respectfully requested.

F. Rejection of Claims for Double Patenting

Claims 1-5, 11-24, and 27 are rejected under the judicially created doctrine of double patenting over claims 1 and 2 of U.S. Patent No. 6,693,130 (the "'130 patent") or claims 1-9 of U.S. Patent No. 6,531,506 (the "'506 patent"). According to the Action, administering sEH inhibitors to persons with hypertension would inherently practice the invention as claimed. Applicants amend in part and traverse.

Claim 1 as amended recites that the methods of the invention do not apply to persons with hypertension. Accordingly, the claims of the '130 patent and of the '506 no longer relate to claim 1 as amended and the claims dependent thereon. Claim 27 has been canceled without prejudice.

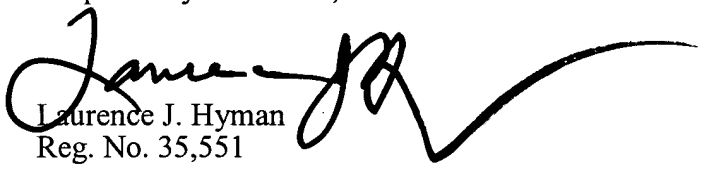
Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, he is invited to telephone the undersigned at 415-576-0200.

Respectfully submitted,



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Attachments: as stated

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METHOD OF TREATING IMMUNOLOGICAL DISORDERS MEDIATED BY T-LYMPHOCYTES

Legal status (INPADOC) of **WO0023060**

WO F	**9924371 W**	(Patent of invention)
PRS Date :	2000/02/10	
PRS Code :	ENP AU	
Code Expl.:	ENTRY INTO THE NATIONAL PHASE IN:	
CORRESP. PATENT D.:	2000 13170	
KD OF CORRESP. PAT.:	A	
FILING(F)/PUBLIC(P):	F	

PRS Date :	2000/04/27
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Code Expl.:	+ DESIGNATED STATES
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PRS Date :	2000/04/27
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PRS Date :	2000/06/21
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Code Expl.:	EP: THE EPO HAS BEEN INFORMED BY WIPO THAT EP WAS DESIGNATED IN THIS APPLICATION

PRS Date :	2000/09/08
PRS Code :	AK
Code Expl.:	+ DESIGNATED STATES
KD OF CORRESP. PAT.:	A3
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PRS Code :	AK
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Code Expl.:	CORRECTED VERSION OF A PAMPHLET FRONT PAGE

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Code Expl.:	CORRECTION OF ENTRY IN SECTION I
FURTHER INFORMATION:	PAT. BUL. 17/2000 UNDER (81) ADD "ZA"; DUE TO LATE TRANSMITTAL BY THE RECEIVING OFFICE

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Endothelial Dysfunction, the Renin-Angiotensin System, and Nitric Oxide: Impact on Coronary Artery Disease and Therapeutic Interventions

Carl J. Pepine, M.D.
Guest Editor

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Introduction

The Vascular Biology Working Group was formed under the auspices of the University of Florida College of Medicine to bring together researchers from various fields to explore the clinical implications of recent basic research related to the endothelium. A central focus was work dealing with the pathogenesis of cardiovascular disease. The initial meetings of the Working Group were held in March 1994 (North America) and March 1995 (Europe), and annual meetings have refined our understanding of the complex processes that influence the development of atherosclerosis, hypertension, and other vascular diseases. The primary goal of these meetings is to translate basic and clinical research into a message useful to the practicing physician, with the hope that it could impact on adverse outcomes of patients with cardiovascular disease.

This supplement to *Clinical Cardiology* is based on the proceedings of Working Group meetings held in the United States and Europe in late 1996 and early 1997. The nine articles by prominent cardiologists review the current understanding of the endothelium as a mediator of cardiovascular tone and structure. They also discuss interventions that are proposed (e.g., estrogen in postmenopausal women, L-arginine supplementation) and proven [e.g., HMG-CoA reductase inhibitors, angiotensin-converting enzyme (ACE) inhibitors, and others] to improve endothelial function in the coronary circulation of patients with atherosclerosis or hypertension.

To begin, Thomas F. Lüscher, M.D., from University Hospital Zürich and the University of Zürich, Switzerland, establishes the basis for the ensuing discussions with an overview of the biology of the endothelium [PDF]. As he explains, the endothelium produces substances that regulate both relaxation and contraction of blood vessels, and it also contributes to the maintenance of vascular structure.

Next, David G. Harrison, M.D., of Emory University School of Medicine, Atlanta, examines oxidant stress

and endothelial function [PDF]. We now know that oxidation inactivates nitric oxide and likely contributes to many abnormalities of endothelium that characterize atherosclerosis, hypertension, and other disease processes.

The article onthe homeostatic balance between angiotensin II and nitric oxide [PDF] by Gary H. Gibbons, M.D., from Brigham and Women's Hospital in Boston, provides an excellent overview of the balance between vasoconstrictors and vasodilators as well as between growth promoters and growth inhibitors. In this regard, angiotensin II in particular mediates vascular remodeling. Dr. Gibbons indicates that blocking angiotensin II by ACE inhibition may have profound effects on vascular function and structure.

Although recent research has established that endothelial dysfunction of both large and small blood vessels contributes to hypertension, the exact cause of the pathologic disturbance that causes endothelial dysfunction in patients with hypertension has not been defined. Julio A. Panza, M.D, and colleagues at the National Institutes of Health, Bethesda, have conducted a series of investigations designed to clarify the potential contributions of various endothelium-dependent and -independent factors to abnormal endothelial function. He provides an outstanding overview of the relation between hypertension and endothelial dysfunction [PDF] as well as an update on the results of experiments to identify potential mechanisms.

A primary focus of the Vascular Biology Working Group is the role of the renin-angiotensin system and ACE in endothelial function [PDF]. Douglas E. Vaughan, M.D., of Vanderbilt University Medical Center, Nashville, explores this relationship as it pertains to local fibrinolysis, one of the primary endogenous mechanisms for preventing intravascular thrombosis. He provides results of the newest studies showing that ACE inhibition can interrupt the occurrence of acute ischemic events in some populations.

As the inner lining of blood vessels, the endothelium is involved early in the development of atherosclerotic plaque. Plaque disruption causes coronary thrombosis, which is, in turn, the primary mechanism responsible for acute coronary syndromes such as unstable angina, acute myocardial infarction, and sudden cardiac death. The article by Prediman K. Shah, M.D., of Cedars-Sinai Medical Center, Los Angeles, provides an update on the pathogenesis and prevention of plaque disruption and coronary thrombosis [PDF], including the recent concept of plaque stabilization as a potential clinical intervention.

In his discussion of the endothelium as a target organ [PDF], John P. Cooke, M.D., Ph.D., from Stanford University School of Medicine, Stanford, reviews risk factors known to lead to endothelial dysfunction. These range from well-recognized risk factors for atherosclerosis such as hypertension and increased levels of low-density lipoprotein cholesterol to a recently identified factor, asymmetric dimethylarginine (ADMA), an endogenous antagonist of nitric oxide synthase. A number of potential therapeutic interventions, both pharmacologic (ACE inhibitors, lipid-lowering agents) and nonpharmacologic (e.g., antioxidants), have been shown to improve endothelial function. They accomplish this by modifying or reducing the effects of these factors or by decreasing the vulnerability of the endothelium to damage.

Christophe Bauters, M.D., of the University and Cardiology Hospital of Lille, France, presents results of experimental studies performed at his laboratory on the beneficial effects exerted by several vascular

growth factors [PDF], including basic fibroblast growth factor and vascular endothelial growth factor, on endothelial function. These growth factors offer a new avenue for therapy of ischemia in either the limbs or the heart.

The final article by myself examines the potential role of ACE inhibition in clinical myocardial ischemia [PDF]. A number of recently completed or ongoing randomized, clinical trials are reviewed. Results of these trials will provide critical information on the potential benefits of ACE-inhibitor therapy in improving endothelial function.

It is hoped that the publication of this supplement, made possible through an unrestricted grant from Parke-Davis, will further our goal of making the latest research in vascular biology accessible to the practicing physician and provide insights into the basis for clinical interventions to improve outcomes in coronary artery disease.

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Biology of the Endothelium

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Summary: The endothelium releases factors that control vascular relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition. Maintaining the functional integrity of the endothelium, therefore, is critical for the preservation of blood flow and the prevention of thrombosis. This article reviews the primary endothelium-dependent substances that promote either relaxation (e.g., nitric oxide, prostacyclin) or contraction (e.g., endothelin) of blood vessels, including their physiology, mechanism of effect, and role in endothelial dysfunction. Risk factors for cardiovascular disease, such as hypertension, hypercholesterolemia, diabetes, vascular aging, and estrogen deficiency, are discussed in terms of their contributions to endothelial dysfunction, which may be the initial step in atherogenesis.

Key words: atherosclerosis, endothelial dysfunction, endothelin, hypercholesterolemia, hypertension, nitric oxide, risk factors

Introduction

Since the pioneering work of Furchgott and Zawadzki,[1] the endothelium has been recognized as a major regulator of vascular hemostasis. Endothelial cells, as the inner lining of blood vessels, are strategically located between circulating blood and blood cells and the vascular smooth muscle. In a person with a body weight of 70 kg, the endothelium covers an area of approximately 700 m^2 and weighs about 1 to 1.5 kg.[2] Functional integrity of the endothelium is crucial for the maintenance of blood flow and antithrombotic capacity, because the endothelium releases humoral factors that control relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition. Thus, the endothelium contributes to blood pressure control, blood flow, and vessel patency. It is now clear that impaired endothelial function contributes substantially to cardiovascular disorders such as atherosclerosis, hypertension, and heart failure, which lead to hypoperfusion, vascular occlusion, and end-organ damage.

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Physiology of the Endothelium

Endothelium-Derived Relaxing Factors

Stimulation of intact endothelial cells by neurotransmitters, hormones, and substances derived from platelets and the coagulation system causes release of a substance that, in turn, induces relaxation of the underlying vascular smooth muscle (Fig. 1).[1, 3] Furthermore, shear forces generated by circulating blood induce endothelium-dependent vasodilation, which is an important adaptive response of the vasculature during exercise. This endothelium-derived relaxing factor, a diffusible substance with a half-life of a few seconds,[1] has been identified as the free radical, nitric oxide (NO). Nitric oxide is formed from L-arginine by oxidation of the guanidine-nitrogen terminal.[4] The NO-synthesizing enzyme exists in several isoforms in endothelial cells, platelets, macrophages, vascular smooth muscle cells, nerves, and the brain.[5] In endothelial cells, gene expression of NO synthase, although constitutively activated, can be upregulated by shear stress and estrogens. The activity of NO synthase can be inhibited by the circulating amino acid, asymmetrical dimethylarginine (ADMA), which accumulates in patients with renal failure.[6] This observation has been further extended to hypercholesterolemia; increased levels of ADMA were seen in hypercholesterolemic rabbits despite normal renal function,[7] and elevated circulating ADMA was subsequently observed in patients with occlusive peripheral atherosclerotic disease.[8] An inducible isoform of NO synthase exists in vascular smooth muscle and macrophages. When activated by cytokines such as endotoxin, interleukin-1β, and tumor necrosis factor α, this calcium-independent enzyme produces

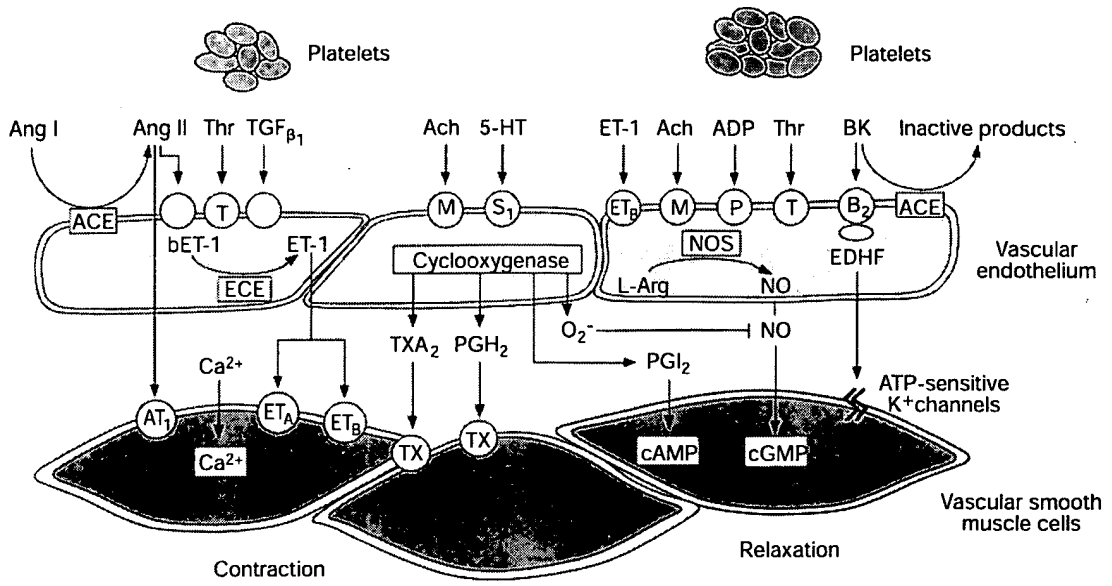

FIG. 1 Vasoactive mediators released by the endothelium. The endothelium produces factors that promote both relaxation (right) and contraction (left). Ang = angiotensin, ACE = angiotensin-converting enzyme, Ach = acetylcholine, ADP = adenosine diphosphate, ATP = adenosine triphosphate, Bk = bradykinin, cAMP/cGMP = cyclic adenosine/guanosine monophosphate, ECE = endothelin-converting enzyme, EDHF = endothelium-derived hyperpolarizing factor, ET = endothelin-1, 5HT = 5-hydroxytryptamine (serotonin), L-Arg = L-arginine, NO = nitric oxide, NOS = nitric oxide synthase, O_2^- = superoxide, PGH_2 = prostaglandin H_2, PGI_2 = prostacyclin, TGFβ_1 = transforming growth factor β_1, Thr = thrombin, TXA_2 = thromboxane A_2. Circles represent receptors (AT = angiotensinergic, B = bradykinergic, ET = endothelin receptor, M = muscarinic, P = purinergic, S = serotonergic, T = thrombin receptor, TX = thromboxane receptor).

large amounts of NO, and hence is activated in inflammatory processes and endotoxic shock.

Endothelium-dependent relaxations due to NO involve formation of cyclic 3',5'-guanosine monophosphate (cGMP) via the soluble enzyme guanylyl cyclase[9] (Fig. 1). Nitric oxide-induced endothelium-dependent relaxation can be pharmacologically inhibited by analogues of L-arginine such as L-N^G-monomethyl arginine (L-NMMA) or L-nitroarginine methylester (L-NAME), which compete with the natural precursor L-arginine at the catalytic site of the enzyme.[5] In isolated arteries, these inhibitors cause endothelium-dependent contractions, whereas in perfused hearts, inhibition of NO formation markedly decreases coronary flow. Local infusion of L-NMMA into the human forearm circulation induces an increase in peripheral vascular resistance. When infused intravenously, L-NMMA induces long-lasting increases in blood pressure. This indicates that the vasculature is in a constant state of vasodilation due to continuous basal release of NO by the endothelium.

In addition to NO, endothelial cells release prostacyclin in response to shear stress, hypoxia, and several substances (see above) that also release NO (Fig. 1). Prostacyclin increases cyclic 3',5'-adenosine monophosphate (cAMP) in smooth muscle and platelets. Its platelet-inhibitory effects play a greater physiologic role than its contribution to endothelium-dependent relaxation. Nitric oxide and prostacyclin synergistically inhibit platelet aggregation, suggesting that the presence of both mediators is required for maximal inhibition of platelet activation.

In the epicardial coronary circulation, inhibitors of the L-arginine pathway do not prevent all endothelium-dependent relaxations, particularly in intramyocardial vessels.[10] Because vascular smooth muscle cells become hyperpolarized during NO-independent relaxations, the existence of endothelium-dependent hyperpolarizing factors has been proposed.[11, 12] However, C-type natriuretic peptide, previously proposed as an endothelium-derived hyperpolarizing factor, does not cause endothelium-dependent hyperpolarization.[13]

Endothelium-Derived Contracting Factors

Soon after endothelium-derived relaxing factor/NO was discovered, it became clear that endothelial cells also can mediate contraction[3] (Fig. 1). Endothelium-derived contracting factors include the 21-amino acid peptide endothelin-1 (ET-1), vasoconstrictor prostanoids such as thromboxane A_2 and prostaglandin H_2, and components of the renin-angiotensin system such as angiotensin II. Three isoforms of the endothelin peptide family exist: endothelin-1, endothelin-2, and endothelin-3. Endothelial cells produce ET-1 exclusively.[14] Translation of messenger RNA generates preproendothelin, which is converted to big endothelin (bET-1) that is further converted by endothelin-converting enzyme (ECE) to the mature peptide ET-1. Four isoforms of this enzyme—ECE-1a, ECE-1b, ECE-1c, and ECE-2—have been cloned.[15, 16] Expression of messenger RNA and release of ET-1 are stimulated by thrombin, transforming growth factor β, interleukin-1, epinephrine, angiotensin II, arginine vaso-

pressin, calcium ionophore, and phorbol ester[14, 17] (Fig. 1).

Endothelin-1 causes vasodilation at lower concentrations but marked and sustained contractions at higher concentrations;[14, 18] in the heart, the latter eventually leads to ischemia, arrhythmias, and death. Intramyocardial vessels are more sensitive to the vasoconstrictor effects of ET-1 than are epicardial coronary arteries, suggesting that endothelin has particular importance in the regulation of flow. Very low circulating levels of ET-1 indicate that most of the peptide is formed locally in the vascular wall. This may be due to the absence of stimuli for endothelin production, the presence of potent inhibitory mechanisms, or the preferential release of endothelin abluminally toward smooth muscle cells.[19] Four inhibitory mechanisms regulating ET-1 production have been delineated: (1) cGMP-dependent inhibition,[17] (2) cAMP-dependent inhibition,[20] (3) an inhibitory factor produced by vascular smooth muscle cells,[21] and (4) inhibition by estrogens via an estrogen-receptor-dependent mechanism.[22] Inhibition of the endothelial L-arginine pathway augments thrombin-induced or angiotensin-induced production of ET-1; conversely, nitrates and atrial natriuretic peptide (which activate particulate guanylyl cyclase) prevent thrombin-induced ET-1 release via a cGMP-dependent mechanism. Endothelin-1 may also promote release of NO and prostacyclin from endothelial cells through ET_B receptors; as a negative feedback mechanism, this process reduces ET-1 production in the endothelium[17] and its vasoconstrictor action in smooth muscle. It is interesting that endothelin inhibits the expression and function of inducible NO synthase.[23]

Two distinct endothelin receptors have been identified: the ET_A- and ET_B-receptors (Fig. 1).[24] Both are G protein-coupled receptors with seven transmembrane domains and are linked to phospholipase C and protein kinase C. Endothelial cells express ET_B-receptors involved in the formation of NO and prostacyclin, which explains the transient vasodilator effects of endothelin when infused into intact organs or organisms. ET_A-receptors and, to some extent, ET_B-receptors mediate contraction and proliferation in vascular smooth muscle. Several endothelin-receptor antagonists have been developed and are currently being clinically evaluated in normal subjects and patients.

The cyclooxygenase pathway also produces endothelium-derived vasoconstrictors. Particularly in veins, but also in the cerebral and ophthalmic circulation, agonists such as arachidonic acid, acetylcholine, histamine, and serotonin can evoke endothelium-dependent contractions that are mediated by thromboxane A_2 or prostaglandin H_2 (Fig. 1).[3] Thromboxane A_2 and prostaglandin H_2 activate the thromboxane receptors in vascular smooth muscle and platelets, thereby counteracting the effects of NO and prostacyclin in both types of cell. In addition, the cyclooxygenase pathway is a source of superoxide anions, which rapidly inactivate NO to form the potent cytotoxic oxidant peroxynitrite.

The endothelium also regulates the activity of the renin-angiotensin system. Angiotensin-converting enzyme (ACE), which converts angiotensin I to angiotensin II, is expressed on the endothelial cell membrane. Angiotensin-converting enzyme is identical to kinase II, which inactivates bradykinin. Angiotensin II can activate endothelial angiotensin receptors; these receptors stimulate the production of ET-1 and other mediators such as plasminogen activator inhibitor.[25] Furthermore, superoxide anion production due to the activation of NADH/NADPH oxidase has recently been linked to angiotensin II-induced hypertension.[26]

Endothelium and Vascular Structure

Removal of endothelial cells by balloon injury invariably leads immediately to deposition of platelets and white blood cells at the site of injury; intimal hyperplasia occurs within days to weeks. This observation suggests that the endothelium regulates vascular structure and that it protects the vessel wall from activation of vascular smooth muscle cells (Fig. 2). Endothelial dysfunction is therefore an important factor in atherosclerosis, restenosis, and hypertensive vascular disease. Vascular structure is determined mainly by vascular smooth muscle cell growth. Endothelial cells may affect vascular structure directly and indirectly. Nitric oxide and prostacyclin inhibit platelet adhesion.[27] Endothelial dysfunction and/or denudation, however, allow platelets to adhere to the vessel wall, where they may cause contraction through the release of thromboxane A_2 and serotonin and may stimulate proliferation and migration of vascular smooth muscle cells via release of platelet-derived growth factor.[28]

Endothelial cells produce growth promoters and growth inhibitors. Under physiologic conditions, the effects of growth inhibitors appear to outweigh those of growth promoters, which may explain why the blood vessel wall is normally quiescent with no proliferation of smooth muscle cells. Heparan sulfates, NO, and transforming growth factor β_1 are potent inhibitors of vascular smooth muscle cell migration and proliferation.[29–31] In contrast, endothelial cells under certain conditions may produce various growth factors, particularly platelet-derived growth factor, epidermal growth factor, and angiotensin II (Fig. 2). These factors may become important in disease states in which the endothelium remains morphologically intact but dysfunctional and may thereby contribute to smooth muscle cell proliferation.

Pathophysiology of the Endothelium

Endothelial Dysfunction: Marker or Mediator?

Endothelial dysfunction is characterized by an imbalance of endothelium-derived relaxing and contracting factors. It may be the cause or consequence of vascular disease and is a hallmark of known cardiovascular risk factors (see below). It is interesting that endothelial dysfunction precedes structural vascular alterations, indicating a protective role of the functionally intact endothelium. While some vessels are particularly prone to developing endothelial dysfunction and atherosclerosis (epicardial coronary arteries, large arteries such as the aorta or iliac artery), others appear to be protected (internal

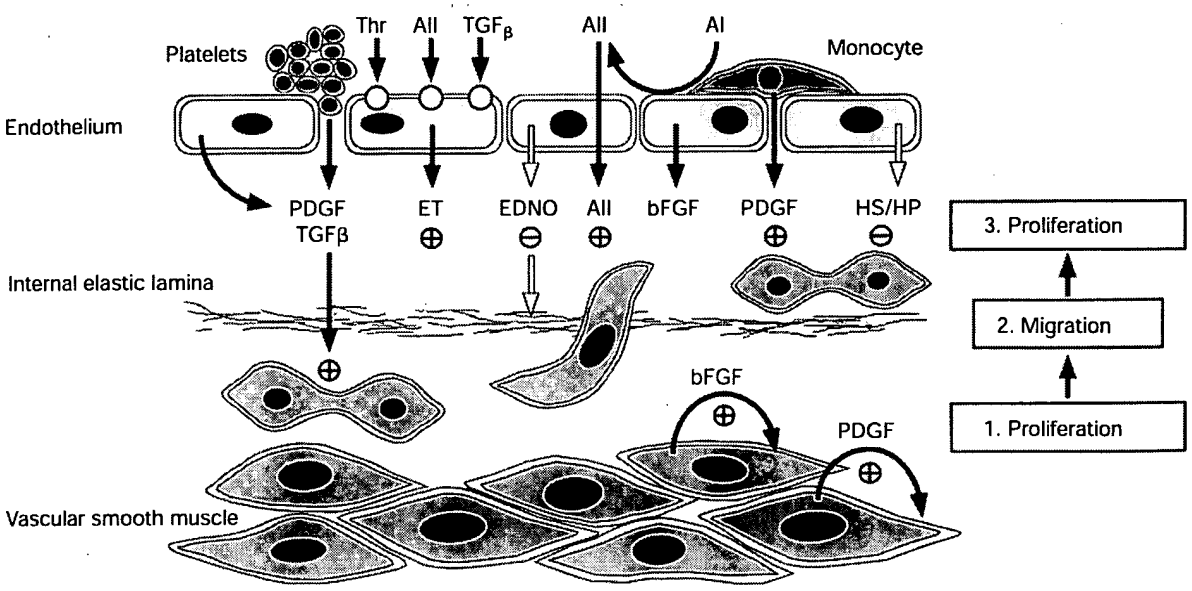

FIG. 2 The endothelium and control of vascular structure. Under normal conditions, the endothelium does not stimulate migration and proliferation of vascular smooth muscle cells. With onset of endothelial dysfunction, platelets and monocytes adhere to the vessel wall, and growth factors are released from these cells as well as from the endothelium. AII = angiotensin II, bFGF = basic fibroblast growth factor, EDNO = endothelium-derived nitric oxide, HP/HS = heparan sulfates, PDGF = platelet-derived growth factor. Other abbreviations as in Figure 1.

mammary artery, brachial artery). This difference may relate to selective alterations due to pulse pressure and/or alterations in endothelial cell function in different areas of the vascular tree. Endothelial cell denudation, however, occurs only in very late stages of atherosclerosis and plaque rupture. These changes in endothelial cell morphology are almost invariably associated with functional alterations and intimal thickening, with accumulation of white blood cells, vascular smooth muscle cells, and fibroblasts and matrix deposition.

Cardiovascular Risk Factors and Endothelial Dysfunction

Hypercholesterolemia: Hypercholesterolemia per se, without atherosclerotic vascular changes, inhibits endothelium-dependent relaxations, which are further reduced in atherosclerosis.[32] It appears that low-density lipoprotein (LDL) is a major determinant of this phenomenon (Fig. 3). Indeed, incubation of isolated coronary arteries with oxidized but not native LDL selectively inhibits endothelium-dependent relaxations to serotonin, aggregating platelets, and thrombin, whereas the response to bradykinin is not affected.[33] A similar diminution of the response can be achieved by pertussis toxin or an inhibitor of NO formation, suggesting defective activation of the L-arginine pathway by G_i protein-coupled receptors.[33, 34] Exogenous L-arginine improves or restores reduced endothelium-dependent relaxation in the presence of oxidized LDL, which suggests that oxidized LDL impairs the activity of NO synthase. The active component of LDL appears to be lysolecithine, which mimics most of the effects of LDL. In vitro experiments in the coronary arteries of hypercholesterolemic pigs have demonstrated selective dysfunction of endothelium-dependent relaxation in response to serotonin and to aggregating platelets and thrombin. Endothelial dysfunction is more extensive in more advanced stages of atherosclerosis. Experiments in the aorta of hypercholesterolemic rabbits suggest that the overall production of NO is not reduced but rather augmented; however, increased production of NO is inactivated by superoxide radicals produced within the endothelium[35] (Fig. 3). Similar observations have been made in rabbits with fully developed atherosclerosis. Under the conditions of both hypercholesterolemia and atherosclerosis, biologically active NO is markedly reduced, a fact also supported by bioassay experiments with coronary arteries of hypercholesterolemic pigs.[36]

Endothelin is activated in atherosclerotic vascular disease. In hyperlipidemia and atherosclerosis, endothelial cell production of endothelin is increased[37] (Fig. 3), while the expression of endothelin receptors is downregulated.[38] A most likely stimulus for the increased endothelin production is LDL, which increases endothelin gene expression and endothelin release from porcine and human aortic endothelial cells[39] (Fig. 3). Vascular smooth muscle cells, particularly those that migrate into the intima during the atherosclerotic process, also produce endothelin. In cultured vascular smooth muscle cells, endothelin can be released by growth factors such as platelet-derived growth factor and transforming growth factor β_1 and by vasoconstrictors such as arginine vasopressin.[40] Hence, several mediators involved in atherosclerosis stimulate vascular endothelin production, perhaps explaining why plasma endothelin levels are increased and correlate positively with

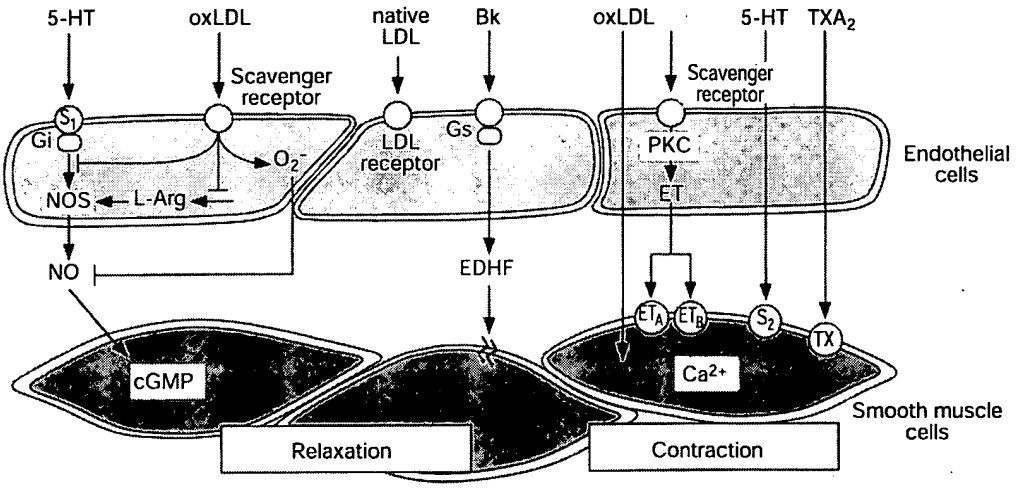

FIG. 3 Endothelial dysfunction in hyperlipidemia and atherosclerosis. The major contributor is oxidized low-density lipoprotein (oxLDL), which, by activating scavenger receptors, impairs the activity of the L-arginine-NO pathway. The mechanism may involve inactivation of G_i proteins (G_i), decreased intracellular availability of L-arginine (L-Arg), and increased breakdown of NO by superoxide (O_2^-). oxLDL further activates endothelin (ET) gene expression and production via protein kinase C (PKC). Other abbreviations as in Figure 1.

the extent of atherosclerotic lesion formation.[37] Furthermore, unstable lesions removed from coronary arteries by atherectomy exhibit marked staining for ET-1.[41] Thus, local vascular endothelin may contribute to both abnormal coronary vasomotion in patients with unstable angina, which may be stimulated by ischemia or thrombin, and to vasoconstriction and the proliferation of vascular smooth muscle cells observed in atherosclerosis.

Hypertension: Endothelial dysfunction in hypertension may contribute to an increase in peripheral vascular resistance (in small arteries) or to vascular complications of the disease (in large and medium-sized conduit arteries). In most models of hypertension, high blood pressure is associated with reduced endothelium-dependent relaxation.[3] Endothelial dysfunction is more prominent in some blood vessels than in others and appears to occur as blood pressure rises; thus, endothelial dysfunction is a consequence rather than a cause of hypertension. In hypertensive subjects, acetylcholine causes paradoxical vasoconstriction of epicardial coronary arteries. The mechanism of endothelial dysfunction differs in various models of hypertension. In the spontaneously hypertensive rat model of genetic hypertension, the activity of the enzyme NO synthase is markedly increased but inefficacious, probably due to increased inactivation of NO by superoxide anion[42] (Fig. 4). In addition, the endothelium of spontaneously hypertensive rats and ren-2 transgenic rats produces increased amounts of prostaglandin H_2, which offsets the effects of NO in vascular smooth muscle and platelets. Whether or not this occurs in humans is uncertain; however, in the forearm circulation of patients with essential hypertension, infusion of a cyclooxygenase inhibitor such as indomethacin enhances vasodilation to acetylcholine.[43] In contrast, salt-induced hypertension is associated with a marked impairment of endothelial NO synthase activity[44] (Fig. 4). Plasma levels of endothelin remain normal in most patients with hypertension except in the presence of renal failure or atherosclerosis. Increased local vascular production of endothelin, however, is likely; because most of the peptide is released abluminally,[19] plasma levels of endothelin do not necessarily reflect local tissue levels. Vascular endothelin production is reduced in the spontaneously hypertensive rat but increased in angiotensin II-induced hypertension in Wistar-Kyoto rats. In the latter model, functional ECE activity is also increased.[45] However, endothelin by itself does not appear to cause hypertension.[46]

Vascular aging: Aging is a physiologic process associated with an increase in cardiovascular morbidity and mortality even in the absence of known cardiovascular risk factors. This may be related to cellular changes in response to increased oxidative stress[47] or to other factors such as impaired release of vasoactive mediators. In most studies, endothelium-dependent relaxations decrease with aging. In humans, the increase in coronary flow induced by acetylcholine infusion lessens with age.[48] Recent studies have demonstrated that the decline in endothelium-dependent relaxation may be related to a decrease in basal[49] and stimulated[50] release of NO and to reduced expression of the endothelial NO synthase gene. Vascular function is preserved with aging, however, in some arteries such as the femoral artery (Fig. 5).[49] Although plasma levels of endothelin increase with age, the response to endothelin decreases, presumably due to downregulation of receptors in most vessels. Similarly, aging heterogeneously affects functional ECE activity, which may increase in some but not all arteries.[49]

Diabetes: Elevated glucose levels in patients with diabetes cause endothelial dysfunction. The underlying mechanism may involve increased synthesis of endothelin[51] and/or impairment of the L-arginine-NO pathway. Recent studies have

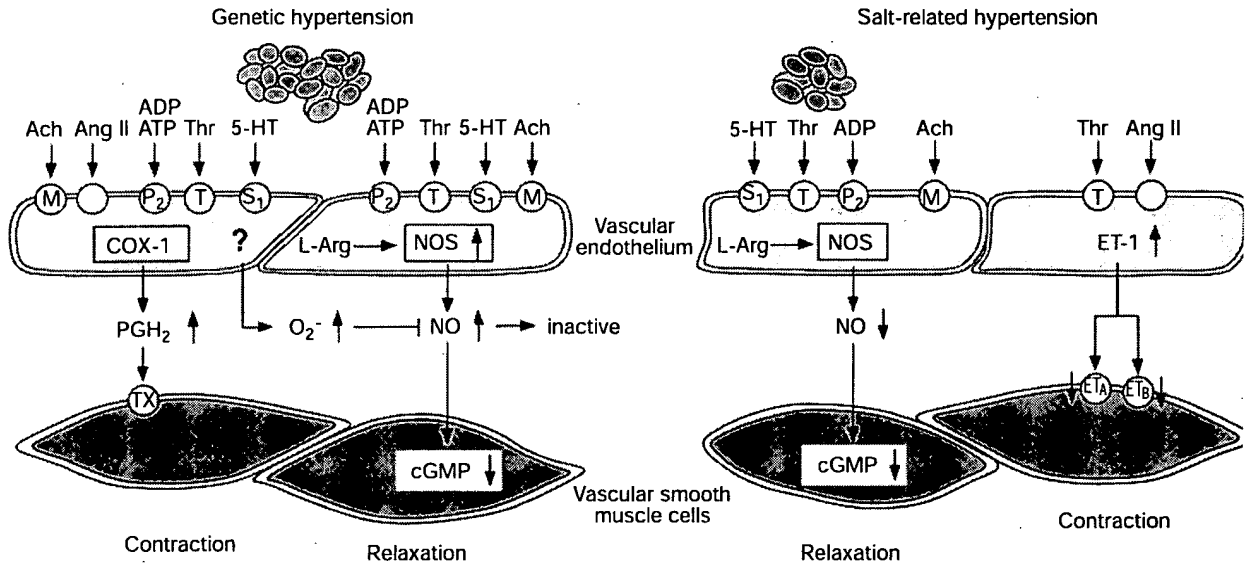

FIG. 4 Endothelial function and hypertension. In spontaneously hypertensive rats (SHR; left), nitric oxide synthase (NOS) activity is increased, but the biological activity of nitric oxide (NO) is reduced, possibly due to inactivation by superoxide (O_2^-). In addition, the production of thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) via cyclooxygenase (COX-1) is increased. In contrast, in salt-related hypertension (Dahl rats, Sabra rats, Doca® salt hypertension), NO production is reduced. Production of endothelin (ET-1) is increased in Dahl or Doca® salt hypertension but reduced in SHR. Doca® = desoxycorticosterone acetate. Other abbreviations as in Figure 1.

shown that elevated glucose concentrations increase expression of NO synthase and production of superoxide anion in vitro.[52] Vascular dysfunction due to high glucose levels appears to be mediated in vivo by vascular endothelial growth factor via an NO synthase-linked pathway.[53]

Estrogen deficiency: Estrogen is an important modulator of vascular function. Estrogen replacement therapy is associated with a decreased risk of cardiovascular morbidity and mortality in postmenopausal women.[54] Accordingly, male gender is considered an independent risk factor for coronary artery disease. Estrogen modulates NO synthase activity and the formation of NO in vitro and in vivo. Estrogen deficiency is associated with endothelial dysfunction[55] and increased circulating levels of endothelin.[56] Endothelin can be inhibited by estrogen in vitro[22] and in vivo.[56]

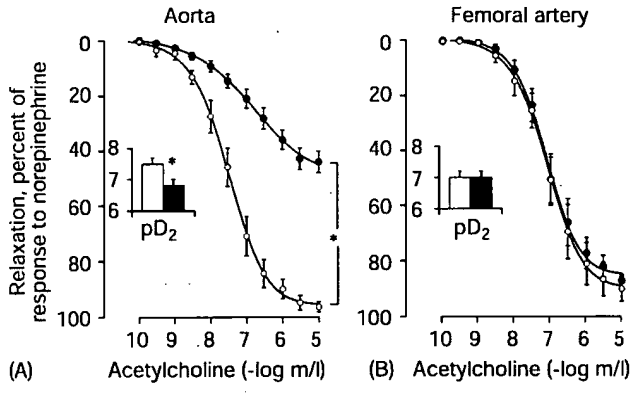

FIG. 5. Endothelial function and vascular aging. Aging impairs NO-mediated endothelium-dependent relaxations to acetylcholine in the aorta of Wistar rats (A), whereas endothelial function in the femoral artery is maintained (B). The different responses indicate an anatomical heterogeneity in the aging process of the endothelium. • Old (n = 6), ○ young (n = 8). Reprinted with permission from Ref. No. 49.

Clinical Implications

Experimental and clinical evidence suggests that endothelial dysfunction is a major determinant for the development and progression of cardiovascular and renovascular diseases. A major goal of therapy in patients with these diseases should be to improve or preserve endothelial function. Furthermore, since endothelial dysfunction occurs prior to structural vascular changes, therapy should be initiated early in patients at risk (e.g., familial hypercholesterolemia, hypertension). Prevention or correction of endothelial dysfunction in cardiovascular disease with agents targeting the endothelium, such as ACE inhibitors, HMG-CoA reductase inhibitors, and estrogen, is likely to improve the clinical outcome in these patients.

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Endothelial Dysfunction in Essential Hypertension

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Summary: In the last decade, significant advances have occurred in our understanding of the presence and nature of endothelial dysfunction in a number of cardiovascular conditions, including hypertension. Endothelium-derived nitric oxide (NO) is recognized as an important mediator of endothelium-dependent vascular relaxation, and a defect in the endothelium-derived NO system—possibly decreased synthesis and/or release of NO by endothelial cells—is now known to cause the abnormal response to acetylcholine in hypertensive vessels and to account at least in part for the increased vascular resistance observed in hypertension. Extensive research by our laboratory and others to determine the nature of the defect in the NO system has found that the defect is not related to decreased availability of L-arginine, the NO precursor, or to a defect at the muscarinic receptor level or a specific G protein-dependent intracellular signal-transduction pathway; nor is it related to extracellular inactivation of NO by superoxide anion. These findings have contributed to our understanding of endothelial dysfunction in essential hypertension and have pointed out distinctions between the mechanisms leading to this vascular abnormality in hypertensive and hypercholesterolemic patients. While the exact nature of the NO system defect in hypertension is still to be clarified, the vasoconstrictive and proatherogenic effects of endothelial dysfunction probably contribute to the cardiovascular complications associated with elevated blood pressure. Continued research targeted at the identification of the precise mechanism(s) responsible for endothelial dysfunction in hypertension may lead to the development of novel therapeutic strategies to reduce the vascular complications associated with the hypertensive process.

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Introduction

Endothelial dysfunction contributes to the underlying disease process of a number of conditions, including essential hypertension, hypercholesterolemia, atherosclerosis, diabetes mellitus, congestive heart failure, and pulmonary hypertension. Over the last decade, extensive research has focused on determining not only the presence but also the nature of endothelial dysfunction in patients with conditions associated with premature development of atherosclerosis. It is now known that certain aspects of the endothelial dysfunction of patients with essential hypertension differ from those of patients with other risk factors. Continuing research is attempting to determine the precise mechanism of endothelial dysfunction in various cardiovascular conditions.

Studies of endothelial dysfunction generally evaluate the vascular responses to endothelium-dependent (agents that need the presence and integrity of the endothelium to exert their relaxing effect on smooth muscle) and endothelium-independent (agents that bypass the endothelium to act directly on the smooth muscle) vasodilators such as acetylcholine and nitroglycerin or sodium nitroprusside, respectively. Because acetylcholine has been the most widely used endothelium-dependent vasodilator, it is important to recognize that its effect differs depending on the vascular bed: in epicardial coronary arteries, acetylcholine elicits a vasodilator response when the endothelium is intact; however, this is transformed into paradoxical vasoconstriction in patients with atherosclerotic coronary artery disease (CAD).[1] In resistance vessels, however, the response is vasodilation, even in conditions associated with endothelial dysfunction. Because blood pressure is largely regulated by resistance vessels (microcirculation), an appropriate assessment of endothelial dysfunction in hypertension involves measuring changes in blood flow and vascular resistance, while changes in the diameter of coronary and other conductance arteries reflect endothelial dysfunction related to atherosclerosis.[1,2]

Thus, meaningful assessment of endothelial regulation of vascular tone in hypertensive patients requires measuring the

effects of endothelium-dependent and endothelium-independent vasodilators on the systemic microcirculation. The forearm perfusion technique provides an excellent model for this purpose because it permits the study of the human microcirculation in vivo without the confounding effects related to activation of systemic counterregulatory mechanisms of vascular homeostasis. This is achieved by measuring the forearm blood flow response (which is dependent on changes in the caliber of small resistance vessels) to pharmacologic agents directly infused into the forearm circulation via a catheter placed in the brachial artery. Blood flow is measured noninvasively by means of strain gauge plethysmography before and during the infusion of incremental doses of the agonists.

Endothelial Dysfunction and Hypertension

Over the last decade, several studies conducted at the National Institutes of Health have assessed endothelium-dependent vascular relaxation in patients with essential hypertension, defined as chronically elevated blood pressure (> 145/95 mmHg) without any apparent underlying cause in patients who had been treated with antihypertensive medication(s) for several years.[3,4]

In our initial study, we showed that although basal forearm blood flow was not different between patients with essential hypertension and normotensive control subjects, the response to acetylcholine, an endothelium-dependent vasodilator, was significantly blunted in hypertensive patients (Fig. 1).[4] However, the response to sodium nitroprusside, an endothelium-independent vasodilator acting directly on smooth muscle cells, was preserved in patients with hypertension. The abnormal response to acetylcholine was not due to presynaptic inhibition of norepinephrine release by adrenergic nerve terminals, since significantly blunted responses to acetylcholine were observed in patients with hypertension following blockade of alpha-adrenergic receptors,[3] indicating that the abnormal response is independent of sympathetic activity.

Similar abnormal responses to acetylcholine or methacholine with preserved responses to sodium nitroprusside have been shown by many other investigators using different vascular models.[5–9]

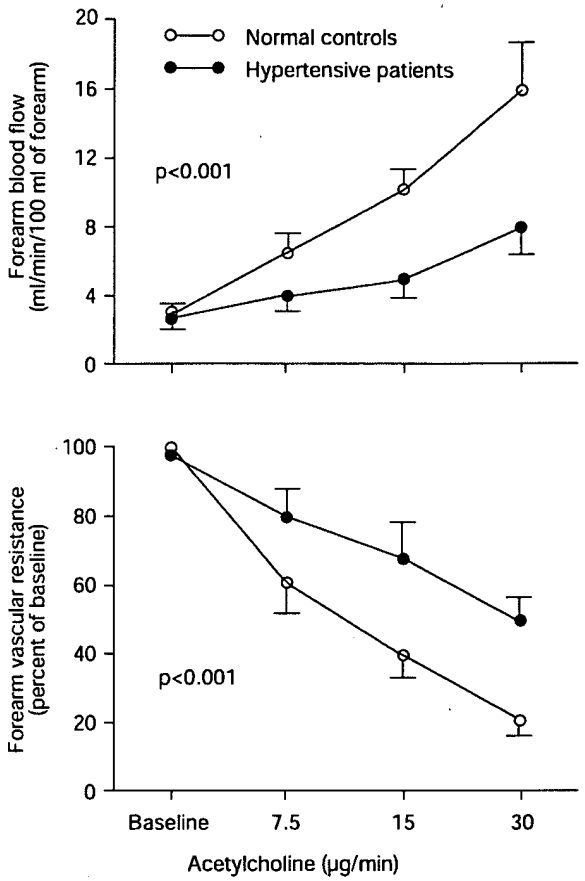

FIG. 1 Vascular responses to administration of acetylcholine in 10 normal controls and in 11 patients with hypertension. The response to acetylcholine was significantly reduced in patients with essential hypertension compared with controls. Adapted from Ref. No. 4 with permission.

Nature of Endothelial Dysfunction in Hypertension

Maintenance of vascular tone and blood flow by the endothelium is complex, involving many substances and an interplay among numerous cellular mechanisms.[10] Thus, while an impaired vasodilator response to acetylcholine indicates the presence of endothelial dysfunction, it does not identify the nature of the dysfunction. As noted above, the abnormal response to acetylcholine in patients with hypertension does not result from a different presynaptic inhibition of norepinephrine release. The role of the endothelium-derived relaxing factor nitric oxide (NO), which is synthesized from L-arginine,[11] has received extensive study. In particular, it has been hypothesized that defects in the synthesis and/or release of NO may play an important role in the endothelial dysfunction of hypertensive vessels.

Basal release of NO has been shown to be critical for the maintenance of vascular tone. Thus, when the synthesis of NO in vivo is blocked by inhibitors of NO synthase, significant vasoconstriction ensues.[12] Because NO has a very short half-life, these findings are consistent with continuous basal release of NO as an important part of the physiology of the vascular system. This role of NO in vascular homeostasis has also been demonstrated in both the resistance and conductance arteries of the coronary vascular tree.[13,14] It is important to realize that, in addition to regulation of vascular tone, NO has other important antiatherogenic actions (including inhibition of platelet aggregation, monocyte migration, and lipid oxidation). Therefore, it is not surprising that a decreased bioavailability of NO has been shown in the coronary arteries of patients with atherosclerosis or its risk factors.[14,15] Furthermore, that hypercholesterolemia and other risk factors impair

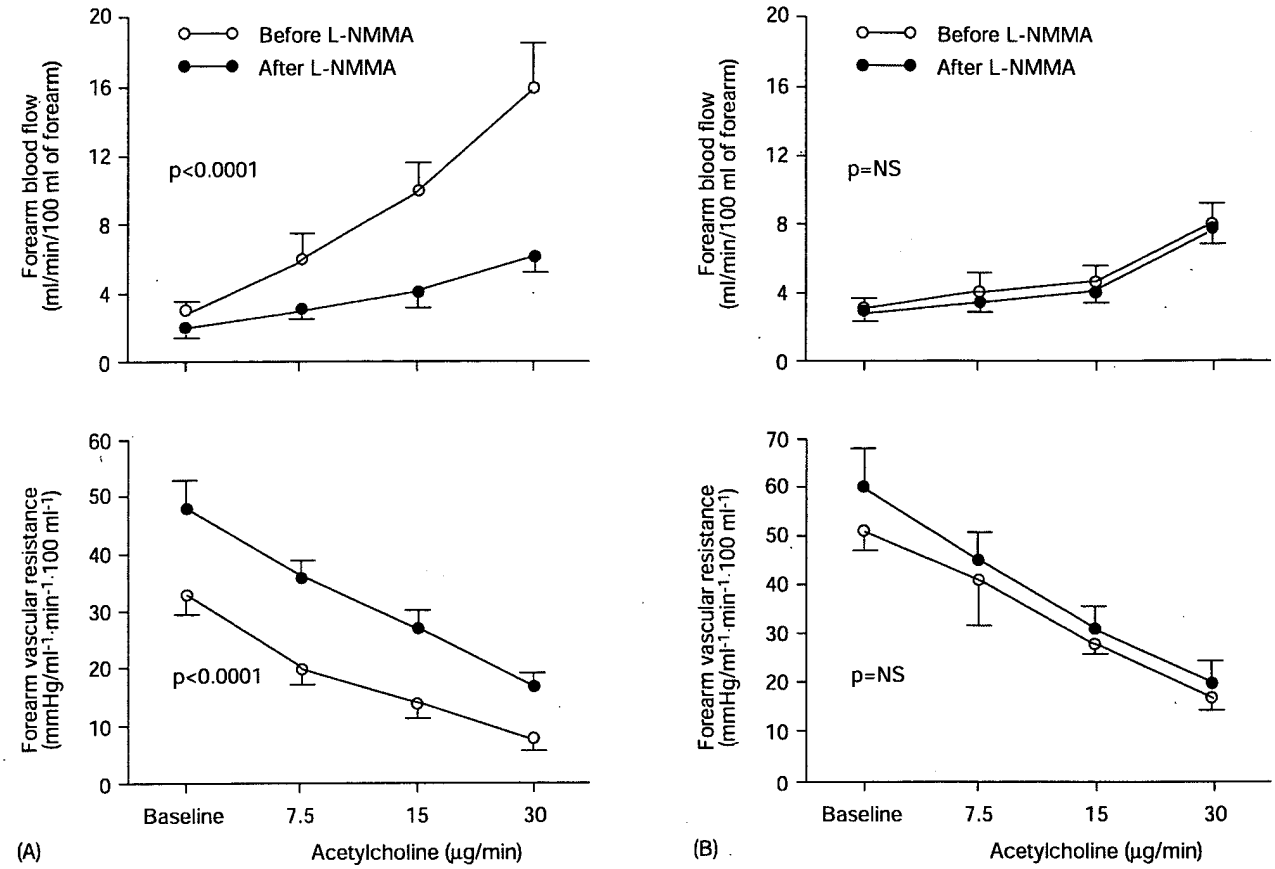

FIG. 2 Effects of L-NMMA on vascular responses to acetylcholine in 10 normal controls and 11 patients with hypertension. Infusion of L-NMMA effectively blunted the vasodilator response to acetylcholine in normotensive controls (A), but no significant change occurred in the already blunted response to L-NMMA in hypertensive patients (B). L-NMMA = N^G-monomethyl-L-arginine. Adapted from Ref. No. 4 with permission.

endothelial function even before the development of atherosclerosis suggests that NO dysfunction is at the very core of the pathogenesis of the atherosclerotic process.

Abnormal Nitric Oxide Activity in Essential Hypertension

The arginine analogue N^G-monomethyl-L-arginine (L-NMMA), which inhibits endothelial synthesis of NO, has been used to investigate the role of endothelium-derived NO in the abnormal endothelium-dependent vasodilation observed in patients with hypertension.[4, 16] In normotensive controls, basal release of NO has been indicated by a reduction in blood flow and an increase in vascular resistance during infusion of L-NMMA into the brachial artery.[4, 12, 16] Patients with hypertension also showed a vasoconstrictive response to infusion of L-NMMA, but the effect was significantly blunted, indicating that much less NO is produced/released by hypertensive vessels in the basal state.[4, 16]

The infusion of L-NMMA into the brachial artery effectively blunted the vasodilator response to acetylcholine in normotensive controls (Fig. 2A), whereas in patients with hypertension no significant change occurred during infusion of L-NMMA in the already blunted response to acetylcholine (Fig. 2B).[4] This finding suggests that NO contributes little to the vasodilator effect of acetylcholine in hypertensive vessels.

Reduced basal and stimulated NO bioactivity has also been shown in patients with hypercholesterolemia, atherosclerosis, or risk factors for atherosclerosis.[14, 15, 17] In hypercholesterolemic patients, the vascular responses to acetylcholine are blunted, and there is no significant change in this response during infusion of L-NMMA.[17] The reduction in endothelium-dependent vasodilation in these patients thus appears to be related to an attenuation in stimulated NO bioactivity.

Potential Defects in the Nitric Oxide System Contributing to Its Reduced Activity

The impaired NO activity demonstrated in patients with essential hypertension could be related to one or more abnormalities along the NO system. As mentioned previously, NO is formed using the amino acid L-arginine as a substrate in response to a variety of physiologic and pharmacologic stimuli, and is broken down primarily by superoxide anions originating both intracellularly and extracellularly.[18] Several mechanisms that may potentially account for decreased vascular activity of NO have been investigated in patients with essential

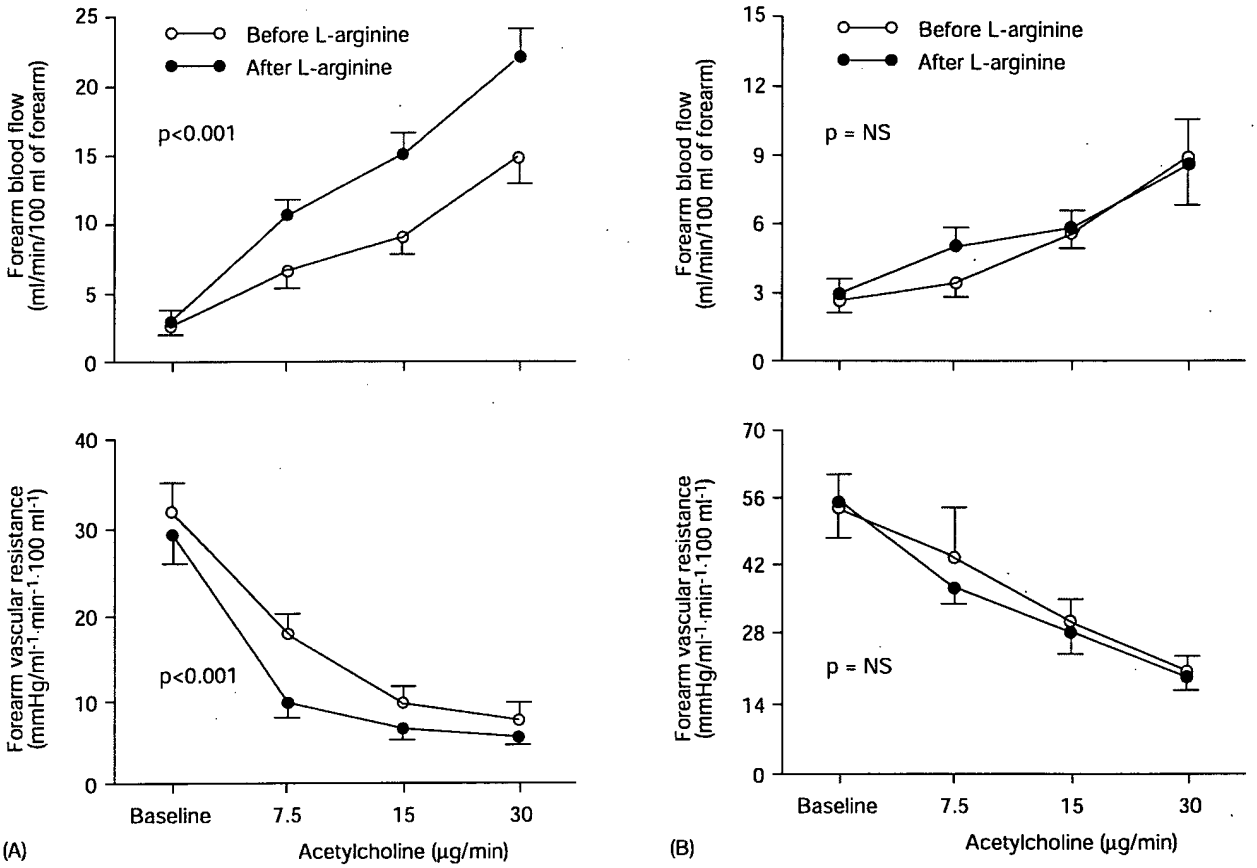

FIG. 3 Effects of L-arginine on vascular responses to acetylcholine in 12 normal controls and 14 patients with hypertension. The response to acetylcholine following L-arginine infusion in normotensive controls was augmented (A); in contrast, little change in response to acetylcholine was observed (B) following L-arginine administration to hypertensive patients. Adapted from Ref. No. 19 with permission.

hypertension in an effort to identify more precisely the nature of endothelial dysfunction that characterizes hypertensive vessels.

Substrate availability: One potential defect in the NO system that could contribute to the decreased bioactivity of NO is a decrease in the availability of its precursor, L-arginine. Studies of the vasodilator response to acetylcholine in normotensive controls showed that infusion of L-arginine into the brachial artery augmented endothelium-dependent vascular relaxation (Fig. 3A).[19] This effect apparently was specific for acetylcholine and L-arginine, as there was no difference in the response to sodium nitroprusside before and after administration of L-arginine and no change in the response to acetylcholine following administration of D-arginine, the isomer of L-arginine. In contrast to the hypothesized improvement in the endothelium-dependent vasodilator response to acetylcholine, little change was observed following L-arginine administration to patients with hypertension (Fig. 3B). These findings indicate that the defect in the endothelium-derived NO system in hypertensive vessels is likely not due to decreased availability of its precursor, L-arginine.[19]

Muscarinic receptor defect: Another postulated defect in the endothelium-derived NO system that might contribute to decreased NO activity involves an abnormality of the muscarinic receptor, which is stimulated by acetylcholine and methacholine. Some evidence has suggested that atherosclerotic coronary arteries with abnormal responses to acetylcholine may demonstrate normal vasodilation in response to substance P, a nonmuscarinic, endothelium-dependent vasodilator [20,21] acting on a different endothelial cell receptor, a tachykinin receptor.[22,23]

It was therefore hypothesized that, if the defect in NO activity of the hypertensive vasculature were located at the level of the muscarinic receptor, the response to substance P would be similar between patients with hypertension and normotensive controls. However, forearm blood flow studies showed a significant reduction in blood flow and vascular resistance responses to substance P in patients with hypertension compared with normotensive controls (Fig. 4). In fact, a correlation was observed between the responses to substance P and acetylcholine,[24] even though the two agonists elicit endothelial responses via different receptors. Moreover, similar responses to substance P during infusion of L-NMMA between normotensive controls and patients with hypertension indicated a reduced NO contribution to substance P-induced vasodilation in patients with hypertension. These findings

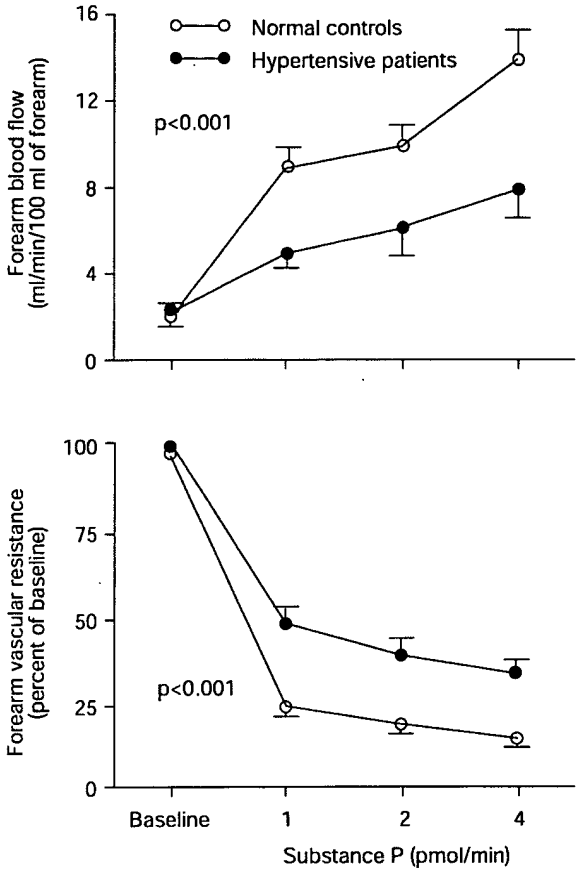

FIG. 4 Vascular responses to substance P in eight normal controls and eight hypertensive patients. A significant reduction in forearm blood flow and vascular resistance response was observed in hypertensive patients compared with normotensive controls. Reprinted from Ref. No. 24 with permission.

suggest that the cause of endothelial dysfunction in patients with hypertension is not limited to a defect at the muscarinic receptor level, but is related to a broader abnormality of endothelial cells.

Signal-transduction pathway defect: Previous studies in animal models of hypercholesterolemia have shown that, early in the disease process, only some endothelium-mediated responses are blunted. As the atherosclerotic process advances, a more generalized defect in endothelium-dependent responses is observed.[25,26] More specifically, investigations using pertussis toxin (a selective inhibitor of certain G proteins) have demonstrated that initially only endothelium-mediated responses that require the activation of pertussis toxin-sensitive G proteins are abnormal, while those utilizing pertussis toxin-insensitive pathways are preserved. Later in the course of disease, responses mediated by pertussis toxin-insensitive G proteins also become affected, although receptor-independent endothelial responses are still intact. Eventually, as the vascular disease progresses, all endothelial responses, even those not mediated by stimulation of cell surface receptors and subsequent activation of G proteins, are abnormal. These observations suggest that the endothelial dysfunction in dyslipidemia may progress through different stages from a relatively selective defect in specific intracellular signal-transduction pathways to a more generalized abnormality of the endothelial cell.[27] These findings have been recently confirmed in patients with hypercholesterolemia without clinical evidence of atherosclerosis.[28]

Based on these observations, it was hypothesized that a similar selective defect in signal transduction was responsible for the impaired endothelial vasodilator function of patients with hypertension. Therefore, in a group of hypertensive patients, the responses to acetylcholine and bradykinin were compared with those obtained in a group of normotensive controls. In contrast to the findings in hypercholesterolemic patients, a significant reduction in forearm blood flow and responses to both acetylcholine and bradykinin was observed in patients with hypertension compared with normotensive controls (Fig. 5).[29] Thus, patients with hypertension had blunted responses to bradykinin similar to their responses to acetylcholine and substance P, indicating that endothelial dysfunction in hypertension is likely due to a more generalized

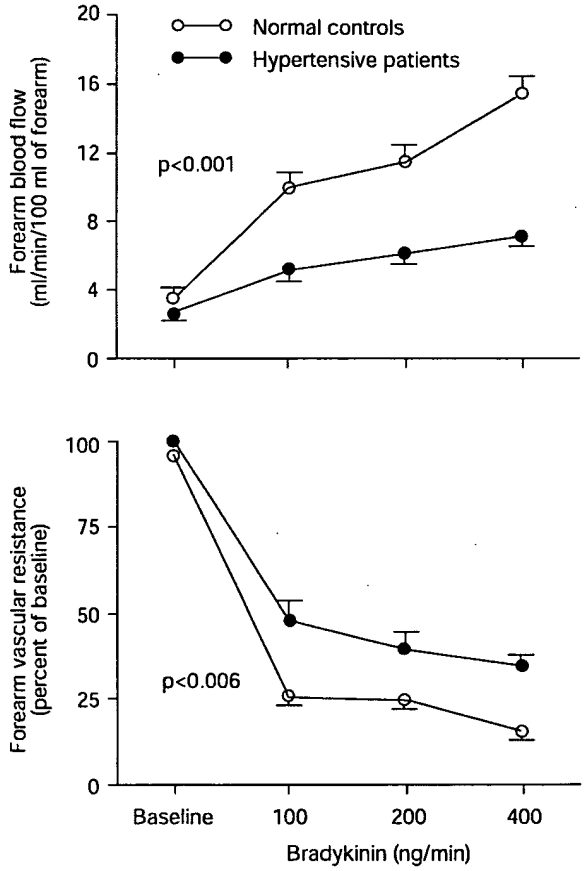

FIG. 5 Vascular responses to bradykinin in 12 normal controls and 10 hypertensive patients. As with substance P, a significant reduction in forearm blood flow and a significant increase in vascular resistance was observed in hypertensive patients compared with normotensive controls. Reprinted from Ref. No. 29 with permission.

abnormality within the endothelial cell rather than a defect in a single G protein-dependent intracellular signal-transduction pathway.

Destruction of nitric oxide by superoxide anion: A principal mechanism of NO inactivation is by superoxide anions produced by various radical-generating systems.[18,30] Superoxide dismutase is a superoxide anion scavenger that may thus block the inactivation of NO.[10,30] Observations from animal models of hypercholesterolemia suggest that excess generation of superoxide anion may be responsible for increased inactivation of NO resulting in impaired endothelium-dependent vascular relaxation in atherosclerotic vessels.[31–33] Similar observations have been reported in different models of hypertension, suggesting that a similar mechanism may be operative in this condition.[34,35] If this were the case in hypertensive patients, then administration of superoxide dismutase would be expected to improve their impaired response to acetylcholine. However, vascular responses to acetylcholine were similar before and after administration of copper/zinc superoxide dismutase in both hypertensive patients and normotensive controls.[36] It must be noted that this form of the enzyme (the only one available for intravascular infusion in humans) protects only against extracellular degradation of NO due to its poor intracellular penetration. Therefore, although these results provided evidence that the defect in the NO system is not due to extracellular inactivation of NO, one cannot rule out the possibility that enhanced production of oxygen-free radical species formed within the intracellular space may contribute to a decreased bioavailability of NO.

An important intracellular source of superoxide radical is the xanthine oxidase system, which can be blocked by administration of oxypurinol. In an animal model, administration of oxypurinol normalized production of superoxide anion and improved acetylcholine-induced relaxation in hypercholesterolemic but not in normal vessels.[31] These findings suggest that endothelial cell production of superoxide anion may inactivate endothelium-derived NO, leading to endothelial dysfunction.

We recently conducted an investigation in which acetylcholine-induced vascular relaxation in hypercholesterolemic patients was improved following oxypurinol administration,[37] an observation in agreement with the results in hypercholesterolemic animal models. However, in patients with hypertension, there was no difference in the response to acetylcholine before or after administration of oxypurinol.[37] These observations suggest that the xanthine oxidase system does not significantly contribute to the endothelial dysfunction of patients with hypertension.

Effect of Antihypertensive Treatment on Endothelial Dysfunction

The observation that induction of hypertension in animal models resulted in impaired endothelium-dependent vasodilation[38] led to the hypothesis that effective antihypertensive therapy may normalize or at least improve endothelial vasodilator function. Indeed, in spontaneously hypertensive rats, treatment with an angiotensin-converting enzyme (ACE) inhibitor or a calcium-channel blocker reduced blood pressure and improved endothelial dysfunction in resistance vessels.[39] Long-term, but not short-term, treatment with an ACE inhibitor or a calcium-channel blocker improved endothelial dysfunction in a rat model of NO-deficient hypertension.[40] These studies further suggested a beneficial effect on endothelial function with antihypertensive treatment.

Studies in humans of the effects of antihypertensive treatment, including studies specifically related to the use of ACE inhibitors, have yielded negative results.[41–43] One study, however, did demonstrate an acute improvement in endothelium-dependent forearm vasodilation with ACE inhibitor treatment,[44] although it must be pointed out that in this study vasodilator responses to acetylcholine and sodium nitroprusside were measured only 1 h after oral administration of captopril, which may explain the discrepancy with the aforementioned studies using longer-term ACE inhibitor therapy.

The role of the renin-angiotensin system in controlling blood pressure and vascular reactivity has long been known. The more recent finding of a local renin-angiotensin pathway in many tissues, including blood vessels,[45,46] suggested that treatment with an ACE inhibitor might improve endothelial function. The recently published Trial on Reversing Endothelial Dysfunction (TREND) study assessed responses to endothelium-dependent and -independent vasodilators in the coronary circulation of patients with CAD.[47] To remove confounding factors, only normotensive (or controlled hypertensive) patients without evidence of severe dyslipidemia or heart failure were enrolled. In large coronary arteries following 6 months of quinapril treatment, the vascular response to acetylcholine significantly improved compared with placebo. It must be emphasized that history of hypertension was not a predictor of improved endothelial function with quinapril, suggesting that the observed vascular effect was independent of the antihypertensive action of the ACE inhibitor. Because atherosclerosis is an important vascular complication of essential hypertension, one might speculate that chronic antihypertensive therapy with ACE inhibition may result in reduction of atherosclerotic disease in hypertensive patients by virtue of its beneficial effect on endothelial dysfunction of the macrovasculature (e.g., epicardial coronary arteries) despite the previously discussed negative effect on endothelial function of the microvasculature (e.g., forearm resistance vessels). This possibility is purely speculative at the present time and deserves further investigation in properly designed trials.

Conclusion

Over the last several years, significant progress has been made in our understanding of endothelial dysfunction in patients with hypertension. Not only has impaired endothelium-dependent vasodilation been demonstrated in patients with hypertension, but we now know that it is related largely to an abnormality in the endothelium-derived NO system.

Research has eliminated several potential defects in the NO system, including decreased availability of the NO precursor L-arginine, a defect at the muscarinic receptor level or in a single G protein-dependent intracellular signal-transduction pathway, and certain forms of inactivation of NO by superoxide anions as being responsible for impaired endothelium-dependent vasodilation in hypertensive patients. Important differences have been encountered in the responses to certain pharmacologic agents between hypertensive and hypercholesterolemic patients, suggesting that the mechanism(s) leading to the syndrome of endothelial dysfunction may be specific for each condition.

Irrespective of the specific defect in the NO pathway, the proatherogenic and vasoconstrictor effects of endothelial dysfunction probably contribute to the cardiovascular complications associated with elevated blood pressure. Treatment of the underlying pathophysiologic process of endothelial function has the potential for improving clinical outcome in patients with hypertension. In this context, continued research to identify the mechanisms responsible for endothelial dysfunction in hypertension is warranted for the eventual design of more rationalistic therapies.

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